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## Olive Oil polyphenols can be Useful to Prevent Aging-Associated Neurodegeneration

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### Introduction to Alzheimer's Disease

Alzheimer's disease (AD), the most common form of dementia affecting a large proportion of aged people in the developed countries, is considered a protein misfolding disease, and, as it is the case of other pathologies belonging to this group, at the present lacks effective therapy. The key histopathological sign of AD is the presence, in several brain areas, of intracellular neurofibrillary tangles (NFT) of hyper phosphorylated tau, of extracellular amyloid deposits (A $\beta$ ) found in diffuse and senile plaques and around cerebral vessels and of dystrophic and degenerating neuritis [1]. A $\beta$  plaque formation is considered relatively specific to AD pathology; however, NFTs are found associated with other disorders as well [2]. Functional alterations and behavioral deficits that characterize AD are thought to result primarily from the presence of plaque deposits whose main component is a polymeric fibrillar form of the 42 amino acid peptide (A $\beta$ 42) generated by proteolysis of the membrane amyloid precursor protein (APP) [3]. Among the different targets explored to treat AD, anti-A $\beta$  approaches directed toward A $\beta$  clearance and/or decreasing its production have shown promising results in animal models of AD [4].

More recently, the interest in deciphering the relation between plaque burden, tissue functional impairment and neuronal death has focused the importance, as the main toxic species to neurons, of the oligomeric pre-fibrillar assemblies originating at the onset of fibril growth [5]. Accordingly, the research of treatments able to delay AD occurrence and to relieve its symptoms has shifted from the development of molecules interfering with fibril growth to that of molecules able to counteract the appearance of toxic oligomeric intermediates.

Focusing dietary regimens associated with a reduced risk of AD in the aged population can be useful to find molecules exploitable for AD prevention and therapy. Mounting evidence supports the beneficial effects of the Mediterranean diet (MD) in preventing age-related dysfunctions, cancer, neurodegenerative diseases and in attenuating AD-like pathology and cognitive deterioration. In particular, MD appears to be effective against mild cognitive impairment and its conversion to AD [6].

### Olive Polyphenols and the Mediterranean Diet: Valuable Tools against Neurodegeneration

#### The mediterranean diet provides many health benefits

Many available data and population studies suggest that greater adherence to MD is of particularly remarkable for its health benefits; in particular, in addition to displaying antidiabetic, anticancer, antimicrobial, anti-inflammatory and anti-dyslipidemic effects [7], it enhances cognitive function and reduces the risk of developing mild cognitive impairment and of its conversion to AD [6,8]. These findings have been better related to the presence, in the MD, of the extra virgin olive oil (EVOO) and its polyphenols, as shown by several population studies such as the PREDIMED-NAVARRA study [9-11] and by a number of clinical trials. The latter include a study carried out in Australia and New Zealand (ACTRN12613000602729), showing that a cause-effect relationship does exist between MD and aging-dependent impairment of cognition [12]; another study, the Prevention con Dieta Mediterranea Nutrition Intervention Trial (ISRCTN35739639), led to conclude that a MD supplemented with olive oil or nuts is associated with a significant improvement of cognitive behavior in a population of aged people [13].

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Finally, the PREDIMED-NAVARRA randomized trial indicated that a MD intervention modulates the way genetic factors influence cognition [14]. Overall, these population studies and clinical trials support the idea that the MD, particularly the prolonged intake of its polyphenols, provide consistent and effective protection against the risk of aging-associated neurodegeneration [15,16].

The beneficial effects of the MD against the development of AD symptoms can result, at least in part, from its remarkable content of polyphenols (sometimes also referred to as biophenols) arising from the daily consumption of two typical components, red wine and extra virgin olive oil. The polyphenols found in these aliments, besides their known anti-oxidant effect, show several other beneficial properties (see later). These include the ability to interfere positively with protein aggregation inhibiting self-assembly of misfolded proteins/peptides into toxic amyloid oligomers and fibrils [17-19] and to affect cell proteostasis in several ways, including the modulation of protein degradation by the ubiquitin/proteasome and/or the autophagic pathways (see later). Actually, plant, notably olive oil, polyphenols have been shown to possess beneficial effects against AD, whose histopathological signature is the presence of extracellular amyloid aggregates of the A $\beta$  peptide and of intracellular tangles of the tau protein and the ensuing pathologic symptoms [18,20-23]. A possible beneficial effect of olive polyphenols against Parkinson disease has also been proposed [24]. Olive oil polyphenols have also been shown to be effective against insulin resistance and impaired glucose regulation, two main symptoms underlying type 2 diabetes. Considering that increasing evidence supports a strong link between the main symptoms of diabetes (mainly type 2) and AD-associated neurodegeneration [25,26] it is conceivable that olive polyphenols can interfere with both pathologies at similar molecular levels.

## Olive tree polyphenols

Natural phenolic substances are secondary plant metabolites, a major group of over 8000 plant compounds chemically characterized by the presence of one or more aromatic rings with one or more hydroxyl Substituent's [15]. Plant polyphenols belong to the phytoalexins family, including molecules synthesized by plants, often in leaves, to combat pathogens (bacteria, fungi) or to discourage leaf-eating insects. In the family of Oleaceae, some polyphenols are highly concentrated also in fruits before ripening, discouraging animals to eat fruits before their maturation for seed dispersal. The olive tree (*Olea europaea*) produces a specific battery of polyphenols including flavonols, lignans and glycosides. The glycosides are comprised in the iridoids class; these molecules result from a cyclopentane ring fused to a six atom-membered hetero cycle containing oxygen; when the cyclopentane ring is broken the molecule is referred to as a secoiridoid (Figure 1).

Olive tree polyphenols are found in the lipid and water (as minute droplets) fractions (in the latter case as glycones) of olive oil. They include the phenolic alcohols hydroxytyrosol (HT, 3,4-dihydroxyphenylethanol, 3,4-DHPEA) and tyrosol (p-hydroxyphenylethanol, p-HPEA) and their precursors; the HT ester of elenolic acid, known as oleuropein (OLE), is the main responsible for the strong bitter taste of olive leaves and drupes. Less represented polyphenols are the dialdehydic derivative of decarboxymethyl elenolic acid bound to either HT (3,4-dihydroxyphenylethanol-elenolic acid dialdehyde, 3,4-DHPEA-EDA, oleacein) or to tyrosol (p-hydroxyphenylethanol-

elenolic acid dialdehyde, p-HPEA-EDA, oleocanthal). The latter is responsible for the burning sensation occurring in the throat following EVOO ingestion [27]. Other olive polyphenols include verbascoside, the caffeoylrhamnosylglucoside of HT, the lignans 1-acetoxypinoresinol and pinoresinol. Among these polyphenols, the most represented in olive oil are oleuropein, both in the glycosylated and in the aglycone forms, its main metabolite, hydroxytyrosol, and oleocanthal.

The content of polyphenols in EVOO can vary largely and can reach levels exceeding 60 mg/100 g; it depends on the olive cultivar, the ripening stage of the fruit, environmental factors, the extraction conditions, the systems to separate oil from olive pastes and the storage conditions and time. The latter affect spontaneous oxidation and the deposition of suspended particles.

## Molecular Mechanisms Underlying Protection of Olive Polyphenols against Neurodegeneration

### Modulation of the mTOR/AMPK pathways

Most beneficial properties of EVOO polyphenols, notably OLE, have been associated, in addition to their well-known antioxidant power, to the modulation of several types of signaling pathways involving AMP-activated protein kinase (AMPK) and mammalian target of rapamycin mTOR, whose inhibition favors longevity and reduces inflammatory states [7,28]. As far as AMPK is concerned, it is well known its key role in AD pathogenesis [29]. mTOR is a central regulator of cell metabolism that finely tunes the balance between anabolism and catabolism; it is also one of the most potent upstream inhibitors of autophagy, and this inhibition is removed following phosphorylation by AMPK [30]. However, the modulation by OLE of other neuronal signaling pathways, as it is the case of resveratrol [31-33], cannot be excluded.

### Modulation of cell proteostasis

Polyphenols, including the olive ones, are involved in regulation of cell proteostasis, including the ubiquitin/proteasome pathway and the autophagic flux, the latter under the control, among others, of the mTOR/AMPK machinery (see above) [34]. The proteasome is the major proteolytic complex, and increasing evidence indicates that proteasomal function declines significantly with aging and in aging-related pathologies [35]. Plant polyphenols, including oleuropein, can increase proteasome activity, with increased life span [36], together with the expression of proteasomal subunits and of protein chaperones [37].

Autophagy, notably macroautophagy, is a highly conserved cellular program devoid to degradation of chemically modified, misfolded and aggregated proteins as well as aged or damaged organelles, particularly mitochondria (mitophagy). Autophagy is reduced in aged people and it appears severely impaired in people affected by neurodegenerative pathologies [38] including Alzheimer's and Parkinson diseases [39,40]; accordingly, molecules able to foster autophagy appear as good candidates to combat aging-associated neurodegeneration.

Plant, including olive, polyphenols stimulates remarkably the autophagic flux similarly to other well-known autophagy inducers such as lithium, valproic acid and rapamycin [41]. Activation of autophagy appears as one of the ways polyphenols induce most of their beneficial effects against neurodegeneration [23]. Plant polyphenols can increase chaperone-mediated autophagy, a type

of autophagy, through their reported effect of cell stressors, with activation of the heat-shock response and increased chaperone levels, as it has been shown for resveratrol [42] and other polyphenols, although Hsp90 inhibition by oleocanthal has also been reported [43]. In cultured neuronal cells, we have shown that OLE triggers macroautophagy by increasing intracellular calcium levels with ensuing AMPK activation through dependent protein kinase  $\beta$  (CAMKK $\beta$ ) [44]. A similar mechanism was previously reported for resveratrol [45]. Other authors have shown that resveratrol stimulates AMPK activity through PKA in double transgenic mice [31]. In both cases, the final outcome was mTOR inhibition through phosphorylation of TSC or the mTOR component Raptor [46]. AMPK can also stimulate autophagy directly, by phosphorylation and activation of ULK1, a key component of the autophagic pathway [47]. These data support the idea that autophagy activation by OLE (and presumably other olive polyphenols) proceeds via the  $\text{Ca}^{2+}$ /CAMKK $\beta$ /AMPK signaling pathway coupled with mTOR inhibition, similarly to data reported for other plant polyphenols [48].

Less data on the beneficial effects of other olive polyphenols in biological and animal models are presently available. In the case of oleocanthal, a recent study performed on a human blood brain barrier model (BBA) and on the TgSwDI murine model of AD showed that animals administered for 4 weeks with the molecule displayed enhanced cerebral clearance of A $\beta$  matching significantly reduced plaque load in the hippocampus. The enhanced cerebral clearance resulted both from increased transport across the BBA and from the activation of the ApoE-dependent clearance pathway [23]. As far as HT is concerned, this polyphenol and its acetylated derivative were reported to be protective in rats fed for 7 days with 5 or 10 mg/kg per day of either compound, respectively [49]. Finally, recent experiments reported that HT administration to the C57BL/6 mouse model of AD results in a significant improvement of the Neuro-behavioral dysfunction in terms of spatial reference and working memory, induced by intra cerebral injection of A $\beta$ 42 plus ibotenic acid [10].

### Modulation of the redox status

Oxidative and nitrosative stress can impair the functionality of brain tissue thus resulting one of the abnormalities underlying neurodegeneration; accordingly, the presence of anti-oxidant molecules, such as olive polyphenols can ameliorate the redox status and hence restore optimal neuronal function. Actually, plant polyphenols, including OLE, have been reported to attenuate the oxidative stress not only due to their well-known antioxidant power but, more remarkably, by restoring mitochondrial function following activation of the nuclear factor (erythroid-derived-2)-like 2, (Nrf2) pathway. Nrf2 is a key transcription factor and a master regulator of the antioxidant response element; it is involved in cell protection against oxidant molecules [50] and its activity matches strongly mitochondrial function and biogenesis. Oleuropein reduces the oxidative damage in the substantia nigra of senescent rats by increasing the catalytic activity of antioxidant enzymes thus protecting against dopaminergic neuronal loss [51]; it also improves the mitochondrial activity in the paraventricular nucleus of the hypothalamus through fostering the Nrf2 pathway with increased expression of antioxidant enzymes such as hemoxygenase 1 and NAD(P)H dehydrogenase quinone 1 [52].

The ability of plant polyphenols to restore a correct redox status is of particular significance in PD, where the

substantia nigra dopaminergic cells are exposed to the pro-oxidant products of dopamine metabolism. In particular, 3,4-dihydroxyphenylacetaldehyde (DOPAL), the main dopamine metabolite, induces *in vivo* the loss of dopaminergic neurons in PD thus favoring  $\alpha$ -synuclein neurotoxicity [53,54]. Another product of dopamine metabolism is HT, the main metabolite of oleuropein. Due to its strong anti-oxidant power, HT relieves DOPAL cytotoxicity; it also diminishes NO-induced cytotoxicity in murine dissociated brain cells [55] and protects rat brain slices subjected to hypoxia-reoxygenation [49]. HT has also been reported to protect cultured dopaminergic cells against the toxic insult provided by dopamine and 6-hydroxydopamine. Also in this case, it was reported that cell protection resulted from the HT-induced expression of phase II detoxifying enzymes, notably NAD(P)H quinone oxidoreductase [24]. These data suggest the possible efficacy of HT against dopamine metabolism-related dopaminergic dysfunction and hence in PD therapy.

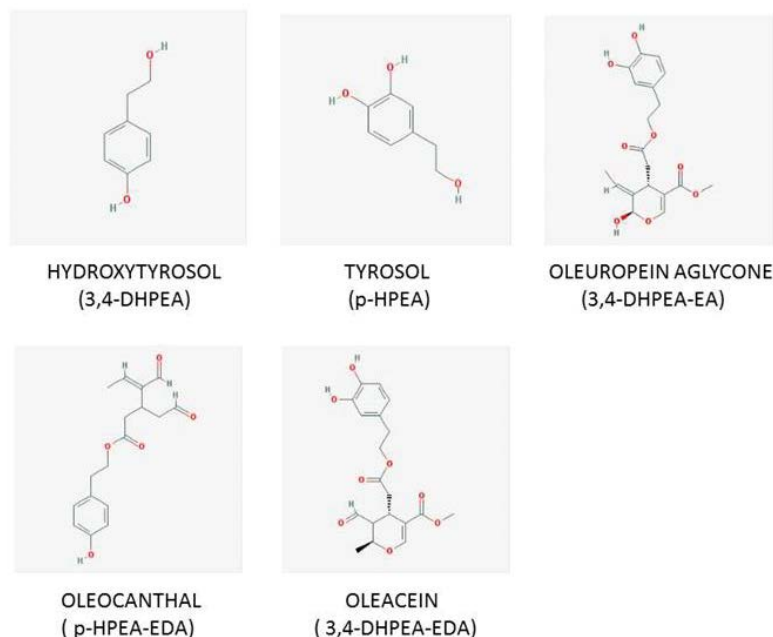
### Epigenetic effects

Plant polyphenols can also counteract aging as well as many of its pathological consequences resulting from aberrant epigenetic mechanisms [56,57]; accordingly, epigenetic issues targeted by diet polyphenols have become an attractive approach for the anti-neurodegeneration power of these substances. In the field of neurodegenerative diseases, the epigenetic modifications have been shown to induce effects similar to those provided by caloric restriction in humans [7]. In this context, we have recently shown that OLE administration down regulates the expression of histone deacetylase 2 (HDAC2), an enzyme known to be up regulated in AD [58], both in the hippocampus and in the cortex of TgCRND8 mice and that such decrease matches a significant increase of histone H3 and H4 acetylation [59]. It must be stressed that histone acetylation has been reported to improve cognitive deficits in animal models of AD and this indication has been proposed as a novel therapeutic strategy against AD [60]. Epigenetic involvement in some of the aforementioned positive effects resulting from OLE administration is further supported by the modifications of the expression levels of effector proteins such as the glutaminyl cyclase, involved in the generation of pyro (3-42) A $\beta$  (pE3-A $\beta$ ) a particularly sticky A $\beta$  derivative considered involved in plaque generation, and some autophagic players such as beclin-1 and LC3. Overall, these data agree with the reported ability of hydroxytyrosol to restore the expression of a number of genes involved in the regulation of memory and survival in transgenic mice [10]. They also add to a large wealth of data that support the ability of many plant polyphenols to induce epigenetic modifications mainly in cancer [61], leading to the proposal of the possibility of an epigenetic diet [62]. Thus, in spite of the limited information on the epigenetic effects of olive polyphenols presently available, modulation of epigenetic flaws by natural polyphenols appears as a promising subject for the development of new drugs based on their molecular scaffold especially designed to combat several chronic degenerative pathologies, possibly including aging-associated neurodegeneration [63].

### Olive Polyphenols Protect Transgenic Mouse Models Against Neurodegeneration

The TgCRND8 (Tg) mice, encoding the Indiana and the Swedish mutations in the APP gene, are cognitively impaired by the age of 3 months [64] and develop a pattern of A $\beta$  deposition





**Figure 1:** Chemical formulas of the aglycone forms of the main polyphenols found in the olive oil.

recalling several aspects of human AD [21]. Small size A $\beta$ 42-immunopositive plaques appear in various brain areas, including cortex and hippocampus, by the age of 3 months. As a function of age, they become small-medium to big in size and radiating in shape and acquire a compact core, becoming numerous and reaching the maximum roughly by 7-9 months of age [21]. In addition, in later stages, from 6 to 12 months of age, of their life this Tg strain exhibits a huge amount of pyroglutamylated A $\beta$  (pE-A $\beta$ ) deposits throughout the cortical and hippocampal areas with a pE3-A $\beta$  load much higher than that of A $\beta$  42 [59].

We previously showed that dietary supplementation of OLE (50 mg/kg of diet) to TgCRND8 mice results in a significant reduction of both soluble and aggregated A $\beta$  and of the astrocyte reaction in AD relevant brain areas with a remarkable attenuation of A $\beta$ -mediated cognitive deterioration [21], LTP restoration and reduction of pE3-A $\beta$ . In addition, in aged mice displaying increased pE3-A $\beta$  in the brain deposits, we found that OLE is active against glutamylcyclase-catalyzed pE3-A $\beta$  generation by reducing enzyme expression and that it interferes with both A $\beta$ 42 and pE3-A $\beta$  aggregation [59].

Similarly, feeding TgCRND8 mice with a mix of polyphenols from olive mill waste water at the total dose of 50 mg/kg of diet resulted in a remarkable improvement in both non-spatial episodic memory and working memory that correlates with the loss of A $\beta$ 42 and pE3-A $\beta$  load in the brain [65]. Altogether, these data show that olive polyphenols are beneficial either when administered as pure components, OLE, or as a mixture and that their effects are comparable.

### Administration of olive polyphenols reverts neurodegeneration in the TgCRND8 mouse model

The importance of autophagy derangement in neurodegeneration was confirmed by our studies carried out in

the TgCRND8 mouse model showing the importance of autophagy activation as a key effect of OLE protection against neurological impairment. When compared with untreated littermates, we found that OLE administration for 8 weeks to these mice activated autophagy, reduced the inflammatory response resulting from the accumulation of amyloid aggregates of the A $\beta$  peptides and the tissue levels of A $\beta$  itself and of its pE3-A $\beta$  derivative following down-regulation of glutamyl cyclase. The OLE-treated mice also displayed a significant reduction of the astrocyte reaction in the affected brain areas, with strong improvement of memory and behavioral performance, that reached the levels recorded in wild-type mice. Finally, the OLE-treated mice displayed increased hippocampal neurogenesis and improved synaptic behavior (as increased LTP) [21,59]. Similar results were reported in C57BL/6 mice, a transgenic model of AD, injected with HT in the cerebral ventriculi. The injected mice showed a remarkable improvement of the neurobehavioral dysfunction, with reversal of dysregulation of several signaling pathways and preservation of the mitochondrial architecture respect to untreated controls [10].

### Drawbacks Associated with the Use of Plant Polyphenols

The pharmacokinetics and pharmacodynamics of olive polyphenols together with the minimal effective doses to be taken to ensure health benefits are poorly investigated. In the case of OLE-fed TgCRND8 mice, it was shown that OLE supplementation could be reduced by 80% without a significant loss of protection whereas a 99% reduction was completely ineffective (Casamenti et al. unpublished data). This lack of knowledge matches a problem associated with the use of olive, and other plant, polyphenols, i.e. their reduced bioavailability due both to incomplete intestinal absorption and to rapid biotransformation. However, many studies have clearly shown that OLE is indeed absorbed by animals and humans and distributed, at least in part, to tissues before

degradation and excretion [66-68]; moreover, we have shown that some OLE metabolites, notably HT, arising mainly from gastric hydrolysis, are found in the brain of TgCRND8 mice after an acute oral administration of OLE, which supports the ability of OLE and/or some of its derivatives, including HT, to cross the blood-brain barrier [59]. We have also shown that the protective effects found in TgCRND8 mice fed with OLE were reproduced when the animals were fed with the same amount of total polyphenols present in olive mill waste water, supporting the idea that different olive polyphenols possess, directly or indirectly, similar protective properties in animals and are similarly absorbed [65]. Overall, in spite of the reduced available information on many aspects of plant polyphenols pharmacokinetics, pharmacodynamics and their metabolic modifications in the organism, also including chemical modifications by the gut micro biota, it is possible to consider that OLE and other olive, and more generally, plant polyphenols are absorbed, though in reduced amounts, and distributed to the whole organism, including the brain, before metabolic modification and disposal [16].

## Conclusion

The identification of novel effective molecules or molecular scaffolds able to contrast protein/peptide aggregation hindering the formation of the plaque deposits widely considered key responsible for neuronal impairment in neurodegenerative diseases is a major, still unmet, medical need for the coming years. The studies carried out in the last decade have highlighted the beneficial effects of the MD against several aging-associated pathologies including neurodegenerative diseases, thus providing strong value to this alimentary regimen. More recently, an increasing body of evidence has started to unravel the molecular basis of these beneficial effects, assigning a key role to the polyphenols enriched in such a diet, particularly those found in red wine (resveratrol) and in the EVOO, key components of the MD. These data are providing a strong rationale and a convincing scientific basis to the health value of the MD in general and, particularly, to the use of nutraceuticals to supplement the dietary intake of these substances, also in the light of their reduced bioavailability. More knowledge is needed to fully confirm these considerations in humans, mainly for what a deeper insight on the molecular and physiological basis of the claimed effects and a better knowledge of all the factors contributing to the effective bioavailability of these substances are concerned. However, the data presently available can be a strong starting point to definitely ascertain the real efficacy of any polyphenols-based nutraceutical treatment aimed at hindering the appearance of aging-associated neurodegeneration and its clinical signs.

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